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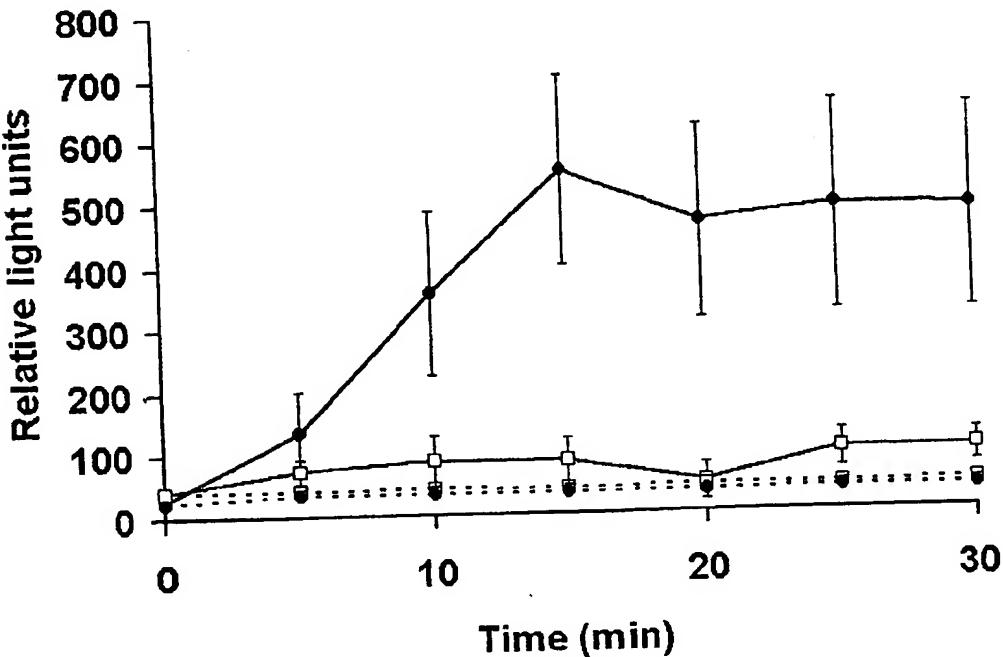
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(54) Title: QUANTIFYING EXPOSURE TO STRESS



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(57) Abstract: The present invention provides an *in vitro* method for quantifying exposure to psychological stress which relies on measuring the retained ability of neutrophils, preferably neutrophils in a whole blood sample, to exhibit challenge-induced superoxide anion production. Using such methodology, coping capacity of individuals for particular psychological stressors may be assessed.



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## QUANTIFYING EXPOSURE TO STRESS

The present invention relates to *in vitro* methods of quantifying exposure to psychological stress in an individual, which may be a human or non-human animal.

5 More particularly, the present invention provides a method of quantifying exposure to psychological stress which relies on measuring the retained ability of neutrophils, preferably neutrophils in a whole blood sample, to exhibit challenge-induced superoxide anion production, i.e. produce a "respiratory burst" in response to *in vitro* activation. Such methodology may also be applied to screen for stress-relieving

10 drugs.

### Background to the invention

Objective, quantitative and practicable measures of psychological stress are pivotal to studies in many branches of vertebrate biology, including wildlife conservation and management, and are also relevant to the farming industry, animal welfare and veterinary services. Techniques for quantifying levels of human stress are important from a medical perspective, but are also of interest for occupational safety and health. An estimated 1 in 10 people in the UK suffer from work related stress

15 (Health & Safety Executive Information sheet: 1/01/EMSU updated February 2002) at a cost of £3.7 billion to society. Stress-related conditions or effects include immune disorders, cardiovascular disease, muscoskeletal and psychological disorders, workplace injury and ulcers.

20 Various methods have previously been applied in an attempt to assess stress responses, including the perceived stress questionnaire (Cohen et al., J. Health & Social Behaviour (1983) 24, 385-396), measurement of cortisol and hormonal levels in blood, urine, saliva and faeces (Beerda et al., Horm. Behav. (1996) 30, 272-279), haematological values (e.g. Millspaugh et al., Can. Field-Nat. (2000) 114, 196-200) and behavioural observations (reviewed by Rushen in The Biology of Animal Stress

25 Basic Principles And Implications For Animal Welfare (2000) ed. Moberg & Mench, Wallingford:CABI Publishing). However, these approaches have various drawbacks,

and may be inappropriate to use in certain situations. The perceived stress questionnaire is subjective, and so cannot provide an objective measure of stress. Furthermore, questions may not always be answered honestly, for example due to cultural reasons, and this measure of stress cannot be used for testing animals or birds. Measurements of cortisol and hormonal levels in, for example, blood and urine, are often used to assess stress in animals, but these measures are not ideal since their levels change throughout the year. There are no absolute terms of reference, and this type of testing is time consuming. Behavioural observations are also often subjective and difficult to quantify.

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More recently, it was shown that exposure of human volunteers to a short term mental stressor leads to increased superoxide anion production being observable in neutrophils present in peripheral blood samples (Ellard et al., *Inter. J. Psychophys.* (2001) 41, 93-100). It has now been found that exposure of both animals and humans to psychological stress can be rapidly and readily quantified by relying on measurement of the retained capacity of neutrophils in peripheral blood samples to produce superoxide anions in response to a challenge by phorbol myristate acetate (PMA), a known chemical-inducer for activating neutrophils (Hu et al., *Cell Signal* (1999) 11, 355-360). By this means, for the first time, it has proved possible to obtain a quantitative measurement of coping capacity of animals and humans for known or suspected psychological stressors.

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- Summary of the invention**
- The method of the invention for quantifying stress is based on direct measures of a cellular response which forms part of the armoury of the immune system. It relies on the ability of individuals to mount a challenge-induced immune response after a potentially stressful event. Each individual's capacity to respond to immune challenge is compared with their own baseline level of immune system activity. After exposure to a known or suspected psychological stressor, superoxide anion production in neutrophils is stimulated *in vitro*, and the capacity of neutrophils to

produce further superoxide is measured, in effect determining the extent to which superoxide production has been diminished by the known or suspected stressful event. The ability of neutrophils to respond to such *in vitro* challenge after a stressful event is defined as the individual's coping capacity. Individuals with a higher coping 5 capacity have a greater potential superoxide production and, physiologically, are better able to cope with bacterial challenge after stress. Therefore coping capacity is an *in vitro* assessment of the individual's current physiological status.

In its broadest aspect, the present invention therefore provides a method for 10 determining whether an individual, which may be a mammal, including a human, or a bird, is experiencing changed physiological status arising from a psychological stressor, the method comprising:

- (a) contacting a test sample comprising neutrophils obtained from said 15 individual with an inducer capable of stimulating superoxide production in neutrophils under conditions suitable for such stimulation;
- (b) determining the increase in superoxide production above basal in said test sample after a time period when neutrophils of the same species in a control sample, which are free or substantially free of stress-induced activation or at least derived from one or more individuals exposed to the same regime minus a factor to be tested as a 20 psychological stressor, will exhibit superoxide production under the same *in vitro* conditions; and
- (c) comparing the increase in superoxide production above basal 25 observed in said test sample with the increase in superoxide production above basal observed in a control sample as defined in (b) above under the same conditions;

wherein lower superoxide production in said test sample is indicative of the effect of a psychological stressor on the individual's physiological status. If need be or 30 desired, the measured superoxide production above basal for each sample may be corrected by reference to the number of leucocytes or neutrophils in the sample (although a range of leucocytes can produce a respiratory burst, neutrophils are

responsible for the majority of superoxide production). As indicated above, residual capacity of leucocytes, or more particularly neutrophils, above basal for *in vitro*-induced superoxide induction can be termed coping capacity.

- 5    The control neutrophils may not be entirely free of stress-induced activation but will necessarily be obtained from the same individual or an individual of the same species prior to exposure of the individual to a suspected or known psychological stressor selected for study. For example, Example 1 illustrates application of such methodology to badgers where the additional stress-inducing effect of transport was quantified compared to mere badger capture based on comparing the residual capacity for challenge-induced activation of neutrophils from transported badgers with such residual capacity of neutrophils from captured but not transported badgers (the controls). Example 2 further illustrates application of the invention for quantifying the stress-inducing effect of handling on wild mammals, e.g. wild mammals as may be found in the UK countryside. Indeed, the invention has wide application in the fields of wildlife conservation, veterinary science and animal husbandry for improving animal well-being, as well as applications to humans as further discussed below.
- 10    The samples employed in a method of the invention may be most conveniently whole peripheral blood samples and hence such a method provides rapid results and can be utilised in the field or laboratory. Such a method can be used to elucidate psychological stressors or to determine whether an individual is suffering from recent or continued exposure to a psychological stressor. As indicated above, most 15    importantly, for the first time the present invention provides a means of quantifying the ability of individuals to cope with known or suspected psychological stressors.

Hence, in a preferred embodiment, there is provided a method for determining the coping capacity of an individual for exposure to a psychological stressor, wherein 20    prior to step (a) in a method as defined above said individual is exposed to said psychological stressor for a time period whereby neutrophils in an individual of the same species who is susceptible to stress induced by said stressor will exhibit

increased superoxide production and wherein the degree of further *in vitro* induced superoxide production in said test sample above basal determined in step (c) is a measure of coping capacity.

- 5     In a variation of the above-described methods of the invention, the control sample is substituted by a second test sample comprising neutrophils derived from a second individual, the tests samples being taken from individuals at the same time point before, during or after subjection to different regimes which it is desired to compare as stressors, e.g. before, during or after different protocols for medical treatment. In 10 this case, superoxide production above basal for each sample will be corrected with reference to the white cell count or neutrophil count. This gives a measure of coping capacity standardised to allow for differences in the number of white cells in the peripheral blood of the selected individuals and thereby enables quantitative comparison of the stress-inducing effect of the two regimes. Such comparison may 15 be carried out at more than one time point and generally samples from a number of individuals subjected to the two different regimes will be tested. Application of such methodology to compare perceived stress and measured decrease of leucocyte coping capacity associated with elective and acute cardio-pulmonary bypass surgery is detailed in Example 6. Such methodology of the invention has many applications for 20 optimising procedures to minimise psychological stress and provides a highly convenient quantitative method for evaluating proposals for alleviating psychological stress associated with procedures known to be stressful to human, non-human mammals and birds.

25     Brief description of the drawings

Example 1 illustrates use of the methodology of the invention to test the prediction that badgers (*Meles meles*) have a lower coping capacity when they are subjected to trapping and then transport than when they are trapped but do not experience the 30 additional stress of transport with reference to the following figures:

Figure 1 shows coping capacity measured as relative light units (solid lines, mean ± SE) in 8 badgers after transport (square), and 8 badgers without transport (circle) as determined by chemiluminescence measurement of superoxide production in whole blood after challenge with PMA. Dashed lines represent basal leucocyte activity.

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Figure 2 shows coping capacity calculated as relative light units (RLU) per  $10^9$  neutrophils  $l^{-1}$ . Solid lines represent coping capacity (mean ± SE) in badgers after transport (square), and without transport (circle). Dashed lines represent basal activity.

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Example 2 illustrates use of the invention to quantify stress induced in small wild animals (wood mice and bank voles) by handling with reference to the following figure:

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Figure 3 shows coping capacity measured in relative light units of handled animals (lower line) compared to non-handled animals (upper line) as determined by chemiluminescence measurement of superoxide production in whole blood samples after challenge with PMA.

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Example 5 illustrates application of the invention to determine if the stress of running a marathon causes a reduction in the ability of the immune system to respond to challenge with reference to the following further figures:

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Figure 4 shows typical data for a single marathon runner obtained using blood samples taken pre- and post-marathon and determining the ability of leucocytes in the blood samples to produce superoxide in response to PMA challenge (filled squares: pre-race control; open squares: response in post-race sample).

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Figures 5(a) shows combined data for 16 marathon runners obtained using blood samples taken pre- and post-marathon and determining the ability of leucocytes in the blood samples to produce superoxide in response to PMA challenge (filled squares: pre-marathon control samples; open squares: post-marathon samples).

Figure 5(b) shows the non-PMA stimulated chemiluminescence measured at the same time points for the same blood samples (filled squares: pre-marathon control samples; open squares: post-marathon samples).

- 5 Example 6 describes in more detail the study indicated above comparing perceived stress and leucocyte coping capacity in two groups of patients, elective patients and emergency (acute) patients pre- and post-cardio-pulmonary bypass surgery. The data for leucocyte coping capacity at 35 minutes post-challenge, obtained using whole blood samples and chemiluminescence measurement, is presented in box plots in Figures 6 and 7 wherein phagocytic capacity per cell per min represents measured relative light units divided by the white cell count and the number of minutes post challenge with adjustment for background activity.
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#### Detailed description of the invention

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A method of the invention may be carried out on neutrophils obtained from an individual in any manner whereby the neutrophils are in a sample suitable for chemical-induced superoxide production but will most preferably for convenience be applied to neutrophils in a whole blood sample. For example, in the case of a human, such a sample may be obtained by a simple finger prick. If need be, blood samples for use in a method of the invention may be treated with an anticoagulant. While a whole blood sample may be utilised directly, with or without an anticoagulant, it will be appreciated that a blood fraction comprising neutrophils may alternatively be employed if desired. For example, a sample comprising isolated leucocytes may be employed.

20 A sample to be tested, directly or after further processing, may be obtained during or shortly after exposure of the individual of concern to a suspected or known psychological stressor. Where one or more samples is taken after exposure to a known or suspected psychological stressor, the initial such sample will generally be obtained as soon as possible. Multiple samples from the same individual may be tested taken at different time points during and/or after exposure to a suspected or

- known psychological stressor, e.g. within 1 to 5 minutes, 10, 15, 20 or 30 minutes of exposure to the suspected or known psychological stressor. Multiple samples may be tested which have been obtained at different time points after exposure to a psychological stressor in order to determine peaking of the stress response and /or the individual's recovery rate. Alternatively, for simplicity and speed, an individual's coping capacity for a known stress factor may be tested at a single time point after exposure to the stress factor. When testing for stress in animals, the sample may be obtained whilst an animal is under anaesthesia.
- 10 A sample to be tested in accordance with the invention may be contacted with any chemical inducer which is capable of stimulating superoxide production in neutrophils. The inducer used in the method may be preferably phorbol myristate acetate (PMA), more particularly, for example, the microbial product phorbol 12-myristate 13-acetate obtainable from Sigma. However, alternative inducers which might be employed are well-known. They include N-Formyl-Met-Leu-Phe (fLMP chemotactic peptide), zymosan, lipopolysaccharide or adrenaline. Suitable lipopolysaccharide is obtainable from the cell wall of a gram negative bacterium, for example *E. coli*. A combination of inducers may be employed, e.g. a combination of PMA and fLMP. A suitable concentration of inducer and period and temperature of induction may be determined if need be by simple experimentation. In the case of PMA, typically it may be added to a whole blood sample at about  $10^{-6}$  M to  $10^{-3}$  M and the temperature of the sample maintained at about 37°C. Superoxide production may be measured at various time points or a suitable single measurement time point may be determined.
- 25 The production of superoxide in response to the inducer may be measured by any known means. Preferably, however, chemiluminescent measurement will be employed for convenience coupled with high sensitivity. For this purpose, a suitable amplifier such as luminol (5-amino-2,3-dihydrophthalazine) or isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) may be utilised to detect superoxide as described by Hu et al., Cell Signal (1999) 11, 355-360. Chemiluminescence may be

conveniently measured using a portable chemiluminometer. In this way, coping capacity for stress factors may be readily quantified even outside of a laboratory. As indicated above, the level of superoxide production may be measured at one time point after the addition of inducer to the sample, or at multiple time points.

- 5      Typically, superoxide production is measured at multiple time points at intervals of between 1 and 10 minutes, for example every 5 minutes, after addition of inducer, over a total time period of between 5 minutes and 1 hour, for example over 30 minutes. Preferably, the level of superoxide production is measured after a time period when control neutrophils of the same species, which are free or substantially 10 free of stress-induced activation, will exhibit maximal superoxide production under the same *in vitro* conditions. This time point can be determined by testing samples obtained from individuals who have not been exposed to a psychological stressor, and measuring superoxide production at various time points after addition of inducer. At the preferred time point, it would be expected that the difference between 15 superoxide production in a sample from an individual exposed to stress, and one who has not been exposed to stress, would be at its greatest. Therefore the test is ideally more sensitive to any changes as a result of stress.

20      The production of superoxide in response to the inducer is compared to the basal superoxide production in the absence of inducer of the same sample, or a further sample taken from the same individual. As indicated above, measured superoxide production in samples may also be divided by the white cell count or neutrophil count to allow for differences in circulating leucocytes in the donors.

- 25      A method of the invention may be used to assess individual responses to the same psychological stressor and compare coping capacities between individuals for a particular psychological stressor. For example, two or more individuals, e.g. two or more humans, may be exposed to a known stressor, and their coping capacity measured at the same time point in accordance with the preferred embodiment of the 30 invention set out above. The results can then be compared to find which of the tested individuals has coped the best with exposure to the psychological stressor. An individual with a lower coping capacity as determined by *in vitro* induced superoxide

- stimulation in neutrophils is less able to cope with the stressor than an individual with a higher coping capacity determined in the same manner after exposure to the same stressor. This method would therefore be of particular use in situations where, after a stressful event, a rapid assessment of the individual's ability to cope is required or where it is desired to select individuals on basis of better ability to cope than others with a recognised psychological stressor. Such a method could, for example, form part of a job selection procedure for human individuals where the job is known to entail exposure to psychological stress factors.
- 5      required or where it is desired to select individuals on basis of better ability to cope than others with a recognised psychological stressor. Such a method could, for example, form part of a job selection procedure for human individuals where the job is known to entail exposure to psychological stress factors.
- 10     A method of the invention may also be used to compare the effects of different stressors on the same individual or group of individuals, for example a sample of individuals from the same species. In this case, the individual's coping capacity in response to each stressor is quantified and compared, in order to determine which is the most stressful. Alternatively, a putative stressor may be tested to determine if it 15    results in a change in the individual's physiological status which is indicative of stress.
- 20     As indicated above, methods of the invention are applicable not only to humans but also non-human mammals and additionally birds. For example, a method of the invention can be applied to farmed animals, such as cattle, pigs, sheep, lambs and poultry, e.g. chickens. Importantly, such a method may be used to assess if farmed animals are suffering from significant stress before slaughter with a view to providing meat products which might be labelled "stress-free". A method of the invention may also be applied to farmed animals with a view to providing a wide 25    range of other products which might be labelled "stress-free" or "obtained from stress-free animals", for example, dairy products such as milk, dried milk, cheese, evaporated milk, condensed milks and ice-cream, wool from sheep and lambs tested in accordance with the invention, fine animal hair from animals such as alpacas, llamas, camels, yaks and goats tested in accordance with the invention and coarse animal hair for brush-making. A method of the invention may be applied to chickens 30    for the purpose of obtaining eggs which might be marketed as coming from "stress-free chickens". Methods of the invention may also be applied to farmed animals to

improve animal husbandry techniques. For example, such methodology might be applied for certification of farms as "stress-free farms". Methods of the invention also find application in wildlife conservation and care as illustrated by the badger study already mentioned above and further described in Example 1 below.

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Methods of the invention may be applied in assessing and improving the ergonomic efficiency of a human working environment. Thus, methods of the invention may have application in office design, furniture design, lighting, colouring and heating of a working environment and design of office equipment such as computers. Methods 10 of the invention may additionally find application in such diverse fields as transport design, design of leisure facilities, e.g. spas and gyms, and shopping facilities to enhance well-being and reduction of stress-factors. They may also find application for certification of animal boarding houses, e.g. for cats and/or dogs, as "stress-free".

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It is also envisaged that assessment of coping capacity in accordance with the invention will have many applications in the field of sports science. Example 5 describes using the methodology of the invention to confirm the expectation that the stress of marathon running reduces the ability of the immune system to respond to challenge as evidenced by decreased leucocyte coping capacity in blood samples 20 taken from marathon runners post-marathon. Similar studies applied to sportsmen and sportswomen taking part in other sporting activities may be of value in improving their performance and well-being.

The invention may also be applied in the field of medicine to assess stress of patients ahead of medical treatment, e.g. surgery, to assess stress associated with medical 25 procedures and in optimising such procedures to minimise stress to patients which may aid recovery and reduce risk of further infection.

In a further aspect, the invention also provides a method of screening for a stress-relieving drug, which comprises administering a test compound to an individual, exposing the individual to a psychological stressor, and measuring their coping 30 capacity using a method according to the invention, and comparing their coping

capacity after administration of the test compound to their coping capacity in the absence of the test compound, wherein an increase in coping capacity after administration of the test compound is indicative of stress-relieving ability of said test compound.

5       The individual used in the above screening method may be a human or animal, for example a mouse, rat, hamster, guinea pig or other mammal. The individual is subjected to a stressor, which may be for example an unpleasant stimulus or stressful situation. In humans, the stressor may be completion of a task under time pressure,  
10      for example Raven's progressive matrices, or observing a stressful event which may be fictitious or real. In animals, the stressor may be exposure to an unfamiliar environment. For example, a rat may be placed in an unfamiliar environment such as the "elevated cross". The stressor may also be social stress, for example stress may be induced in mice by housing them in individual cages and then introducing a  
15      stranger. Other suitable psychological stressors may be identified using a method of the invention for determining exposure to such a stressor.

Suitable test substances which can be tested in the above screening method include combinatorial libraries, defined chemical entities and compounds, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as phage display libraries and antibody products. Typically, organic molecules will be screened, preferably small organic molecules which have a molecular weight of from 50 to 2500 daltons. Candidate products can be biomolecules including, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, or amidification to produce structural analogs.  
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30      A stress-relieving drug identified by a screening method as above may be synthesized and/or formulated into a pharmaceutical composition. Formulation with pharmaceutically acceptable carriers and/or excipients may be carried out using

routine methods in the pharmaceutical art. Thus the manner of formulation will depend upon factors such as the nature of the substance and the condition to be treated. Any such substance may be administered in a variety of dosage forms. It may be administered, for example, orally (e.g. as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules), parenterally, subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques.

Once an individual has been diagnosed as suffering stress by a method of the invention, they may be provided with stress-relieving treatment, for example, they may be administered a known stress-relieving drug. Thus in a still further aspect, the present invention provides use of a stress-relieving drug in the manufacture of a medicament for treating an individual, e.g. a human, who has been identified as suffering stress by a method of the invention. There is additionally provided a method of treating an individual for stress, e.g. by administration of a stress-relieving drug wherein said individual has been identified as suffering from stress by a method of the invention.

A stress-relieving drug for use in such a method may be one that reduces stress, improves or ameliorates the symptoms of stress, or a drug that treats a stress-related disorder. For example, the drug may be a known anxiolytic or antidepressant or an adjunct to such drug therapy, e.g. drugs such as tryptophan. The main classes of anxiolytic drugs are benzodiazepines, for example diazepam and alprazolam; 5-HT<sub>1A</sub>-receptor agonists, such as bupirone; and β-adrenoceptor antagonists, for example propranolol. The main classes of antidepressant drug are tricyclic antidepressants, for example imipramine and amitriptyline; selective serotonin re-uptake inhibitors, for example fluoxetine, fluvoxamine, paroxetine and sertraline; monoamine oxidase inhibitors, such as phenelzine, tranylcypromine, clorgyline and moclobemide; and atypical antidepressants, for example nomifensine, maprotiline, mianserin, bupropion and trazodone.

In a still further aspect, the present invention provides a method of testing the efficacy of a proposed stress-relieving treatment which comprises exposing an individual, e.g. a human, to a psychological stressor in the presence and absence of said treatment and determining their coping capacity as described above. The treatment to be tested may be, for example, handling of a device, gadget or toy designed with the aim of bringing stress relief or exposure to a particular environment, e.g. a smell as in aromatherapy, a pattern of light and/or imagery or sound composed with a view to bringing stress relief. Efficacy of any such treatment will be indicated by an increase in challenge-induced superoxide production indicative of an increase in coping capacity.

As indicated above, where it is desired to compare two regimes as stressors, e.g. two protocols for medical treatment or a known stressful regime plus and minus a proposed means of alleviating stress, then it may be chosen to directly compare coping capacity determined in test samples taken from individuals before, during and after subjection to the two regimes of concern after correction for the number of white cells or neutrophils in each sample.

In a further aspect of the invention, there is provided a device for carrying out a method of quantifying stress according to the invention comprising a portable chemiluminometer together with other components to enable a single device system for quantifying stress. A device of the invention may desirably comprise a portable chemiluminometer linked to a system for analysing the results to provide a stress measurement for each sample.

The following examples illustrate application of the invention to both wild animals and human volunteers.

EXAMPLESExample 1 – Trapping and transport of wild badgers

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As indicated above, this study was carried out to test the prediction that transport of badgers causes substantial additional psychological stress compared to trapping of such animals without transport as reflected in lower coping capacity determined from residual capacity of blood neutrophils for *in vitro* activation.

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Methods*(a) Trapping and transporting badgers*

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Badgers were trapped in Wytham Woods, Oxfordshire, U.K. (for details of the study site and its badger population see Macdonald & Newman (2002) J. Zool. 256, 121-138) in cage traps baited with peanuts in August and November 2001. Badgers were selected because a measure of stress for this species would be immediately useful in studies of its unusual social system (Macdonald & Newman 2002) and its role in the epidemiology of bovine TB. Also, studies, conservation and control of badgers necessitate their capture and handling, so a means of evaluating alternative

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procedures would be helpful. The particular population was selected because their individual life histories have been monitored for 14 years during which the utilised trapping and handling procedures were refined to the highest welfare standards.

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Badger traps were set adjacent to badger setts between 14.00 and 17.00 h. Traps were checked each morning between 06.30 and 07.00 h and trapped badgers were transferred to individual holding cages.

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Animals were then assigned to one of two experimental regimes: sampling at the site of capture, without transport (non-transported, n=8), or sampling immediately after transport (transported, n=8). Transport consisted of a short ride of less than 10 minutes on a trailer pulled by an all-terrain quad bike. While in holding cages, badgers were covered with a cloth. Badgers were anaesthetised, either at their site of

capture or after transport, using an intra-muscular injection of ketamine hydrochloride ("Ketaset," Fort Dodge, U.S.A.) at a dose of 0.2 ml/kg. Processing consisted of measurements of body weight and length, and recording the sex and condition of the badger. Other measurements were also taken as part of the ongoing 5 badger population study. Blood was removed by needle venepuncture of the jugular vein, collected into a tube containing the anticoagulant potassium EDTA (BD Vucutainer Systems, Plymouth U.K.) and taken immediately for PMA challenge. Differential cell counts were made from blood smears fixed in alcohol and stained with May-Grunwald and Giemsa stains (minimum of 100 cells per slide).

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*(b) PMA challenge and measurement of coping capacity*

To measure the background blood chemiluminescence levels, 10 µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial, E G & G Berthold, Germany), to which 90 µl of 10<sup>-4</sup>M luminol (5-amino-2,3-dihydrophthalazine; Sigma 15 A8511) diluted in phosphate buffer was added. The tube was then shaken gently. To measure the chemiluminescence produced in response to challenge, a further tube was prepared as above, but with the addition of 10 µl of the microbial product phorbol 12-myristate 13-acetate (PMA;Sigma P8139) at a concentration of 10<sup>-6</sup>M. The PMA was dissolved in a small amount of dimethyl sulphoxide (DMSO; Sigma D 20 5879) and then diluted to a concentration of 10<sup>-6</sup> M in PBS. For each tube, chemiluminescence was measured for 30 seconds every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold, Germany) for a total of 30 minutes. When not in the chemiluminometer, tubes were incubated at 37°C.

25 Although a range of leucocytes can produce a respiratory burst, neutrophils are responsible for the majority of oxygen free radical production and so leucocyte coping capacity (LCC) was also examined per quantity of 10<sup>9</sup> neutrophils/l. This also provided a method of examining LCC in relation to the potential effects of changes in the number of circulating neutrophils after stress.

30

*(c) Statistical analyses*

To compare differences in coping capacity in badgers with and without transport, multivariate analysis of variance (MANOVA) was used, with the response at each 5 time interval as dependent variables, and transport regime as a treatment variable. This procedure was carried out on SPSS for Windows release 10.0.5. Data were log transformed prior to the analysis to ensure the data met assumptions of multivariate normality (Tabachnick & Fidell (1996), Using Multivariate Statistics, Third Edition, New York: HarperCollins College Publishers). Wilcoxon Signed Ranks Tests were 10 used to compare an individual's coping capacity with its basal response.

*(d) Animal welfare considerations*

The badger population at Wytham Woods is under long-term scientific investigation, and badgers are trapped and transported regularly throughout their lives. Examining 15 transport stress is part of continuing refinement of best practice. Work was carried out under English Nature licence 1991537 and Home Office licence PPL 30/1826.

Results

20 Basal superoxide production in and coping capacity in two groups of animals, transported ( $n = 8$ ) and non-transported ( $n = 8$ ), are shown in Figure 1. Transport significantly reduced coping capacity in badgers ( $F_{7,8} = 4.5, p < 0.05$ ). The difference in coping capacity between transported and non-transported individuals was greatest at 15 minutes ( $F_{1,14} = 8.9, p < 0.05$ ; Figure 1). However, two of the 25 non-transported individuals were caught during a night when ambient temperatures unexpectedly fell below freezing, and these individuals did not show a typical response for this group, and in both cases their coping capacity was no greater than their basal response (Wilcoxon Signed Ranks Test, for both cases  $Z < -1.0, p > 0.1$ ). The transport regime had no effect on basal superoxide production ( $F_{7,8} = 2.5, p >$  30  $0.05$ ). However, to ensure that there was no bias in the LCC results because of individual differences in unstimulated levels of chemiluminescence, we subtracted the basal from the PMA-stimulated values for each animal (PMA-unstimulated).

Transport had an effect on the PMA-unstimulated values from 10 minutes ( $F_{1,14} = 4.1$ ;  $P=0.06$ ) with the difference peaking at 15 minutes ( $F_{1,14} = 9.4$ ;  $P=0.008$ ) and thus showed a pattern consistent with the LCC values described above.

- 5 There were differences in the number of leucocytes between the two groups, with transported individuals having significantly lower numbers of leucocytes (Mann-Whitney  $U = 11.0$ ,  $p < 0.05$ ). In addition, leucocyte composition also differed between the groups: transported animals had a higher percentage of neutrophils (Mann-Whitney  $U = 13.5$ ,  $p = 0.05$ ), and a correspondingly lower percentage of lymphocytes (Mann-Whitney  $U = 7.5$ ,  $p < 0.01$ ). Calculation of LCC per  $10^9$  neutrophils  $\text{l}^{-1}$  gave the same pattern as overall LCC, indicating that activity per neutrophil was greater in non-transported individuals (see Figure 2).

10 These results indicate that stress dramatically reduces the potential of circulating leucocytes to produce free radicals and support that coping capacity is a quantifiable measure of the stress associated with a specific event. The rather uniform depression of the coping response in all transported individuals suggests that this stressor overrode the sources of individual variation that characterised the coping responses of non-transported individuals.

15 **Example 2- Quantification of stress induced by handling of wild small animals**

The purpose of this study was to investigate the stress response to handling in small wild animals, more particularly in wood mice (*Apodemus sylvaticus*) and bank voles (*Clethrionomys glareolus*), by measuring the ability of animals' circulating leucocytes to produce superoxide in response to PMA challenge.

**Methods**

20 ***(a) Experimental design***

One group of animals was handled in the same way as for ecological or behavioural studies and then anaesthetised. Handling brings about a stress response typified by an increased heart rate and attempted escape behaviour and handling is generally

stressful for wild animals. Another group of animals was anaesthetised without handling. Handled animals were removed from their traps by gently tipping them into a plastic bag, and were then handled by their scruff, identified to species and placed in a holding pot and anaesthetised. Non-handled animals were anaesthetised in their traps, without being removed and handled. Initially, the front door of the each trap was opened and the animal inside briefly observed to ensure it was in a suitable condition for undergoing anaesthesia. All traps were then left in a dark, quiet room for at least 30 minutes prior to the experiment to ensure the initial observations did not interfere with the experiment. The non-handled animals served 5 to provide a comparative sample of animals that could be used to determine if handling causes suppression of coping capacity. All animals were anaesthetised using 10 a mixture of oxygen and isoflourane (Mathews *et al.*, Vet. Rec. (2002) 150, 785-787).

The small mammals used in the study were trapped overnight in Longworth live-traps placed in pairs at 5m intervals along hedgerows on two commercial dairy farms 15 in South-West England. Traps were baited with approx 14g of 'Josie' rabbit food (Delta Pet Foods Ltd., Llandovery, Camarthenshire, UK), and apple (approx 5g) as a source of food and water. Laboratory trials revealed that this bait mix is readily consumed by both species. The amount of bait provided meant that food was 20 effectively available *ad libitum* during the period in the trap. Traps were set at 1500 hours and collected at 0800 hours the next morning.

While under anaesthesia, the animal's sex and reproductive status (based on testes status in males and indications of mating or pregnancy in females) was determined. 25 Only males and females were included in the study which were in breeding condition. Animals were then weighed to the nearest 0.1g on a digital balance and their body length from snout to vent measured to the nearest 1mm. Some juveniles were considered to be too small for sampling, and as a result only adult animals (15g and over) were included in the analyses. Animals were also given an individual fur 30 clip mark that could be used to later identify them if they were recaptured. As an index of the resources available to the animal, body weight was used. This was appropriate because, for small mammals, body weight is more closely related to the

level of stored reserves than calculated body condition indices. We also recorded the presence or absence of fleas, since the presence of these relatively common parasites could potentially influence the animal's energy balance. Work was carried out under Home Office Licence PPL 30/1826.

5

Table 1. Details of the forty animals used in the study.

	Wood Mice	Bank voles
Handled	9	16
Not handled	6	9
Weight range, mean (SD)	23.0 (3.1)	22.6 (2.3)
Fleas: present	7	8
absent	8	17

*(b) PMA challenge and measurement of coping capacity*

Blood was removed from the animals by tail venepuncture and 30 µl was collected from each animal in a heparinised capillary tube. To measure the unstimulated blood chemiluminescence levels, 10 µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial, E G & G Berthold, Germany), to which 90 µl of  $10^{-4}$  M luminol (5-amino-2, 3-dihydrophthalazine; Sigma A8511) diluted in phosphate buffer was added. The tube was then shaken gently. To measure chemiluminescence produced in response to challenge, a further tube was prepared as above, but with the addition of 10 µl phorbol 12-myristate 13-acetate (PMA; Sigma P8139) at a concentration of  $10^{-4}$  M. The PMA was dissolved in a small amount of dimethyl sulphoxide (DMSO; Sigma D 5879) and then diluted to a concentration of  $10^{-4}$  M in PBS. For each tube, chemiluminescence was measured every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold, Germany) for a total of 30 minutes. When not in the chemiluminometer, tubes were incubated at 37°C. Leukocyte coping capacity (LCC) was calculated for each individual as the whole blood chemiluminescence measured in response to PMA challenge minus the chemiluminescence measured in unstimulated whole blood.

*(c) Statistical analyses*

To compare differences in coping capacity with and without handling, a multivariate GLM (General Linear Model) was used. The chemiluminescence levels at each time interval were the dependent variables. This approach was chosen because it allowed examination of the effect of the treatment at each time interval, giving more detail on the nature of the effects of the treatment and permitted identification of the time interval at which the treatment effect was greatest. Univariate GLM was also used to examine the factors which significantly affected peak coping capacity. The coping capacity values were log transformed prior to the analyses to meet the GLM requirement of normality of data. A small number of negative values (no smaller than -10) were treated as zeros. This procedure was carried out on SPSS for Windows release 10.0.5.

15     Results

Forty animals were caught for this study and of these 25 were handled (16 bank voles and 9 wood mice) and 15 were not handled (9 bank voles and 6 wood mice) prior to anaesthesia. Handling brought about a marked reduction in LCC, and reduced the variation in LCC (see Figure 3). A multivariate GLM revealed that handling significantly reduced LCC ( $F_{6,33} = 5.1, P = 0.001$ ) and this was the case at all time intervals (5 minutes  $F_{1,38} = 9.9, P = 0.003$ ; 10 minutes  $F_{1,38} = 20.7, P = 0.001$ ; 15 minutes  $F_{1,38} = 26.3, P = 0.001$ ; 20 minutes  $F_{1,38} = 30.4, P = 0.001$ ; 25 minutes  $F_{1,38} = 24.9, P = 0.001$ ; 30 minutes  $F_{1,38} = 22.7, P = 0.001$ ). There was no effect of species in the model, either as a variable on its own ( $F_{6,31} = 0.3, P = 0.9$ ) or as an interacting variable with handling treatment ( $F_{6,31} = 0.4, P = 0.9$ ).

LCC was most variable for handled and non-handled animals after 25 minutes, which was also the peak of LCC. Even given the more uniform response of handled animals, the range of LCC in these individuals after 25 minutes was 220 relative light units (minimum -10, maximum 210, mean 39.3, SD 62.9). A GLM was created using the LCC data at 25 minutes ( $LCC_{25}$ ), which included treatment, treatment  $\times$

- body weight interaction and treatment  $\times$  flea interaction. This model indicated that at LCC<sub>25</sub> there was a significant treatment  $\times$  body weight interaction ( $F_{2,35} = 4.1$ ,  $P = 0.024$ ), but no evidence of a significant treatment  $\times$  flea interaction ( $F_{2,35} = 0.34$ ,  $P=0.91$ ). To explore the treatment  $\times$  body weight interaction further we created
- 5 individual GLMs for handled and non-handled animals. These models indicated that body weight was significantly and positively associated with LCC<sub>25</sub> in non-handled animals (Figure 2;  $F_{1,13} = 5.6$ ,  $P = 0.034$ ), but this relationship was lost when animals were handled ( $F_{1,23} = 0.6$ ,  $P = 0.42$ ).
- 10 This study thus again indicates that leucocyte coping capacity is a quantifiable measure of stress. Handling, a recognised stressor, suppressed the response of circulating leukocytes to PMA. There was no evidence that the level of suppression brought about by handling was related to the animal's body weight, although heavier non-handled animals had a greater peak LCC value. There was variation in the
- 15 response of handled animals to PMA, albeit much reduced in comparison with the response of animals that had not been handled, but no evidence was found to support that the level of resources available to individual animals could explain this variation.

### Example 3 – Observing a fictitious stressful event

- 20 The purpose of this study was to assess the effect of watching a potentially stressful event on human volunteers.

#### Methods

- 25
- (a) *Subjects*
- Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before this study commenced, in accordance with the declaration of Helsinki. 14 subjects all moderately fit and healthy aged between 20-30 participated in the study. Exclusion criteria included suffering from psychiatric illness, respiratory or cardiovascular disease, smokers, or prescription medicine taken within the previous month.

*(b) Design*

The experiments were performed in the afternoon according to highly standardised procedures. Subjects were instructed to avoid exercise or alcohol for 48 hours before the study and be fasting for 2 hours before the study began. Both groups sat quietly 5 for 15 minutes to obtain resting blood pressure, heart rate and resting blood samples. Blood samples were then taken from all subjects 15 minutes before being exposed to either no stress (control conditions) or psychological stress in the form of exposure to the horror film. This time point was designated -15 minutes. Subjects had no prior knowledge as to which group they would be assigned to.

10

8 subjects were exposed to psychological stress in the form of an 83-minute horror film that none of the subjects had previously seen (The Texas Chainsaw Massacre, 1974, directed and produced by Tobe Hooper, Everett Collection, Inc). The remaining 6 subjects acted as a control group. They were instructed to sit quietly for 15 the same length of time (83 minutes), under similar lighting conditions and were given emotionally non-stimulating material to read if they so wished. This consisted of text books, articles from journals and health information leaflets. A repeated measures design was implemented with two groups: the experimental group (watching the horror film) and a control group. Blood samples, heart rate and blood 20 pressure measurements were taken 15 minutes before, and just after (85 minutes) being exposed to either the 'stressful event' or to control conditions.

*(c) Heart rate & blood pressure measurements*

A heart rate transceiver (Polar, Heart rate monitor) was attached directly to the chest 25 and heart rate was monitored. Participants were seated, asked to make themselves comfortable, close their eyes and breathe orthonasally. This procedure was carried out for a period of fifteen minutes, in order to minimise possible stress levels experienced prior to, or upon arrival, at the laboratory. At the end of this period baseline heart rate was recorded and the first blood sample taken (see sample protocol below). Participants were then instructed to either read quietly or in a 30 separate room to watch the horror film. Upon completion of the task heart rate was recorded again, and further blood samples taken. Systemic blood pressure (BP) was

measured using an aneroid sphygmomanometer (Accoson, (Surgical) Ltd, London) and stethoscope (Harvard Ltd, Edenbridge, UK). Finger stick samples of blood 20 µl were obtained at the specified time points.

5       *(d) PMA challenge and measurement of coping capacity*

To measure the background blood chemiluminescence levels, 20 µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial E G & G Berthold Germany), to which 90 µl of 10<sup>-4</sup>M luminol (5-amino-2,3-dihydrophthalazine; Sigma A8511) was added. The tube was then shaken gently. To measure 10 chemiluminescence produced in response to bacterial challenge, 20 µl of phorbol 12-myristate 13-acetate (Sigma P8139) at a concentration of 10<sup>-3</sup> M was added. For each tube, chemiluminescence was measured for 30 seconds every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold Germany) for a total of 30 minutes. When not in the chemiluminometer, tubes were incubated at 37°C. 15 The maximum response to PMA was noted.

*(e) Data analyses.*

Data are expressed as means ± standard deviation from mean. A 2-tailed unpaired t-test was subsequently used to compare the 2 groups at baseline and 85 minutes.

20

Results

Table 2 shows the differences in the response to PMA between the horror film group (n=8) and the control group (n=6). A highly statistically significant (p < 0.05) 25 decrease was found at 85 minutes in the horror film group when compared to the control group. The average heart rate change of +14 for the horror film group represents a 20.6% increase. Their systolic and diastolic blood pressure rose by 10 % and 16 % respectively. The control group showed no such changes.

Max response to PMA (RLU)	-15 minutes Baseline	85 minutes
Control	320 (40)	340 (35)
Horror group	325 (27)	120 (60)*

Table 2: Summary of maximum response to PMA. SD in brackets. \*P<0.05 from control. RLU = Relative Light Units

- 5 Watching a horror movie elicits a psycho physiological arousal which is comparable to Canon's fear flight fight defence reaction, the so called 'stress response' which involves stimulation of the hypothalamus, a change in peripheral resistance and an increase in the release of stress hormones including catecholamines and cortisol.
- 10 This study also shows that stress affected the responsiveness of a peripheral sample of blood. This can therefore be used as an indicator of stress.

An altered responsiveness to PMA indicates *in vivo* changes to the state of leukocyte activation. In a state of stress, it is possible that the leukocytes actually release the contents of their granules (and are thus unable to respond to PMA) having already released the contents of their granules. Such leukocytes would be unable to respond to opportunistic infections thus rendering the host more susceptible to disease, as well as potential tissue damage from a host of proteolytic enzymes and oxygen free radicals.

20 **Example 4 – Stressor task**

**Method**

- (a) *Subjects*
- 25 Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before this study commenced, in accordance with the declaration of Helsinki. 12 undergraduate students (aged 18-23 years) were assigned to either an experimental group (given test) or asked to sit quietly for an equivalent time period. Exclusion criteria included suffering from psychiatric illness, respiratory

or cardiovascular disease, smokers, or prescription medicine taken within the previous month.

*(b) Stressor task*

- 5 The test was a written visual spatial task test comprising 36 questions, on which a 15 minute constraint was imposed (Ravens J. C. Advanced Progressive Matrices, set II, Oxford Psychologists Press, Oxford, 1994). A small finger stick blood sample (Boehringer Mannheim, Soft Clix) was taken before and immediately after task completion. 20 µl of blood was collected in a pipette.

10

*(c) PMA challenge and measurement of coping capacity*

- To measure the background blood chemiluminescence levels, 20 µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial E G & G Berthold Germany), to which 90 µl of  $10^{-4}$ M luminol (5-amino-2,3-dihydropthalazine; Sigma A8511) was added. The tube was then shaken gently. To measure chemiluminescence, 20 µl of phorbol 12-myristate 13-acetate (Sigma P8139) at a concentration of  $10^{-3}$  M was added. For each tube, chemiluminescence was measured for 30 seconds every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold Germany) for a total of 30 minutes. When not in the 15 chemiluminometer, tubes were incubated at 37°C. The maximum response to PMA 20 was noted.

*(d) Data analyses*

- Data are expressed as means  $\pm$  standard deviation from mean. A 2-tailed unpaired t-test was subsequently used to compare the 2 groups at baseline and 15 minutes.

Results

- Table 3 shows the effect of completing a stressor task on leukocyte responsiveness to PMA. The experimental group had significantly reduced responses to PMA ( $p<0.05$ , 30 unpaired t test) compared to controls. Therefore completion of a stressor task diminished responsiveness to PMA.

Max response to PMA (RLU)	Baseline	15 minutes
Control	420 (40)	380 (25)
Experimental Group	380 (30)	160 (70)*

Table 3: Summary of maximum response to PMA. Mean +/- Standard deviation (SD) in brackets. \*P<0.05 from control. RLU = Relative Light Units

5    **Example 5-Determination of the effect of marathon running on the ability of blood neutrophils to respond to PMA challenge *in vitro***

Study Design

10   Sixteen marathon runners were included in the study who during the course of the study undertook the London marathon. Blood samples were taken from the runners the day before the race and immediately following the race. For each blood sample, basal chemiluminescence and chemiluminescence with PMA-challenge was determined in similar manner as described in previous examples. Chemiluminescence 15 measurements were made at 5 minute intervals up to 35 minutes.

Results

20   Figure 4 shows typical data for a single runner. On the day before the marathon, the runner showed response to PMA-challenge as demonstrated by the spike in relative light units (RLU) in the pre-race response line. In contrast, the post-race response line shows no such spike.

25   Most pre-race blood samples showed a peak response to PMA-challenge after 15 to 20 minutes (see Figure 5(a)). The magnitude of the peak response was highly variable. In contrast, the data for post-race samples indicated strong damping of PMA-induced superoxide production compared to the control pre-race samples. The non-PMA stimulated results for the same samples are shown in Figure 5(b). This data is in keeping with the well-known phenomenon that elite athletes are more 30 at risk from certain types of infection than other people and suggests utility of the

invention across a wide range of sporting activities for better understanding of stress in sportsmen and sportswomen and in finding means to ameliorate the effects of stress or reduce stress in such people.

- 5     Example 6- Evaluation of patients' psychological perceptions and immunological responses pre and post cardio-pulmonary bypass surgery (CPB) in two groups of patients a) those who have waited for elective surgery b) patients who are admitted on an emergency (acute) basis.

10    Methods

(a) *Study design*

Ninety patients who were to undergo CPB were assigned to one of two groups. An 'elective' group n = 64 (51 males, 13 females) who had been on waiting list and an 'acute' group n = 26 (22 males, 4 females). Patients were tested on the evening 15 before surgery and at clinic six or seven weeks after discharge using standard measures to assess psychological well-being. Small peripheral blood samples were taken. Neutrophils' 'Current' activity was determined from whole bloods' capacity to reduce nitro-blue tetrazolium (NBT) (%NBT positive cells), and leukocyte coping capacity, from stimulated whole blood, luminol-dependant chemiluminescence.

20

Research was approved by the local health authority ethics committee (University Hospitals Coventry and Warwickshire, NHS Trust), the Walsgrave Hospital Research and Development Committee, the School of Science and the Environment ethics committee (Coventry University) and Cardiothoracic consultant Walsgrave hospital, 25 whose patients formed the basis of this research.

The 90 adults in the study underwent cardiopulmonary bypass surgery for either coronary artery bypass grafting (CABG), mitral valve (MV) surgery or aortic valve (AV) surgery and were recruited on the night prior to surgery. The inclusion criteria 30 were:

- Ability to communicate in both spoken and written English language;
- Informed consent;
- The patient was not involved in any other research or experimental procedures.

The exclusion criteria were:

- 5
  - Previous coronary bypass grafting;
  - An immune deficiency disorder;
  - An inflammatory condition not related to the heart problem.

10   The males of the elected group ranged in age from 38 to 80 (mean 63.61, SD 8.75) and the females of the same group ranged in age from 38 to 81 (mean 64.86, SD 13.29). In the acute patient group, the 22 males were aged between 24 and 80 years (mean 64.86, SD 13.29) and the 4 females were aged between 60 and 81 years (mean 71.75, SD 8.85).

15   Table 4 below shows a comparison of the patient profiles of the two groups observed. The elective group was reduced to 62 patients post-surgery due to death of one patient and one patient still being in hospital with neurological complications. The acute group was reduced to 20 post-surgery due to the death of 5 patients in hospital and one patient opting out of surgery.

Table 4: Preoperative comparative patient profile of both groups.

	Elective Group		Acute Group	
	Males 51	Females 13	Males 22	Females 4
<b>Age</b>				
Range	38 - 80	38 - 81	24 - 80	60 - 81
Mean (SD)	63.61 (8.75)	66.62 (10.67)	64.86 (13.29)	71.75 (8.85)
<b>Type of Surgery</b>				
CABG	37	4	12	3
Valve	10	8	6	1
CABG & Valve	3	1	4	
CABG, Valve & other	1			
<b>Angina Status</b>				
Asymptomatic	6	4	6	
NYHA 1	7	2		
NYHA 2	14	2	1	
NYHA 3	20	3	3	1
NYHA 4	4	2	12	3
<b>Dyspnoea Status</b>				
Asymptomatic	7		1	
NYHA 1	11		2	
NYHA 2	14	2	4	
NYHA 3	11	4	4	2
NYHA 4	8	7	11	2
<b>Coronary Disease</b>				
1 Vessel	3			
2 Vessel	9	2	1	
3 Vessel	30	3	15	2
N/A Valve only	9	8	6	2
<b>ASA Grade</b>				
Healthy	3			
Mild	11	1	2	
Severe	32	11	11	3
Severe/Life threatening	5	1	9	1
<b>Previous Infarct</b>				
None	30	11	8	2
Single	15	2	10	2
Multiple	6		4	
<b>Post Surgery Status</b>				
Alive	49	13	18	2
Still hospitalized	1			
Deceased in Hospital	1		4	1
Opted out/surgery				1

Key To Table 4

CABG = Coronary Artery Bypass Grafting

Valve = Mitral or Aortic Valve replacement or repair

The physicians and the surgeons, on the following scale, preoperatively, graded both  
5 angina and dysnoea status:

NYHA 1 = No limitations of normal physical activity

NYHA 2 = Ordinary physical activity causes discomfort / dyspnoea

NYHA 3 = Moderate to great limitations of ordinary physical activity

NYHA 4 = Unable to perform any physical activity without discomfort / dyspnoea

10 As a gross predictor of overall outcome, post-operatively, an anaesthetist graded each patient on the evening before surgery, using what is termed the ASA grade, as follows:

ASA I = Healthy patient

ASA II = Mild systemic disease – no functional limitation

15 ASA III = Severe systemic disease – definite functional limitation

ASA IV = Severe systemic disease that is a constant threat to life

*(b) Procedure*

Patients were approached by the researcher upon their arrival at hospital or on the  
20 evening prior to surgery (often one and the same time). Briefly, patients were told that the research was to measure their levels of perceived stress and anxiety and the effects this may have on neutrophils before and after surgery. They were additionally told that they would be required to complete a number of simple questionnaires and to provide a small sample of blood from a finger (the equivalent of three drops) on  
25 two occasions, the first being during that evening and the second at the post-operative clinic approximately six weeks after surgery. Any questions were answered and an information sheet was given to each patient and any relatives in attendance.

The researcher then gave the patients time to think about this, returning later with consent forms and asking again if they were willing to take part, explaining that they could if they wished withdraw at any time. If consent was given, demographic data was recorded and patients were asked to complete the questionnaires. A finger stick  
5 blood sample was taken from a cleaned finger using a Soft-Clix™ and sterile lancet and a Gilson pipette was used to collect the blood (3 by 10 µl samples were taken). One sample was placed in 40 µl of luminol in a luminometer test tube. The second sample was mixed with an equal quantity of nitro-blue tetrazolium (NBT) and the third sample was placed in 90 µl of diluting fluid for white cell counting (see sample  
10 protocol below). The wound was dressed if required. Any questions were answered and then the patients were left in peace.

At the post-operative clinic approximately six to seven weeks after surgery, patients were again seen by the researcher and were asked if they wished to continue. If so,  
15 the questionnaires were again completed and blood samples taken again.

*(c) Protocols*

(i) *Measurement of PMA-challenge induced superoxide production in blood samples*  
10 µl of whole blood was mixed with 40 µl of luminol diluted to  $10^{-4}$ M with phosphate buffered saline (PBS) in a luminometer test-tube. The sample was placed  
20 in a luminometer and relative light units (RLU) were recorded after a period of 60 seconds. This initial test was carried out to determine the background activity of the sample so that when calculating coping capacity this background activity could be deducted. 20 µl of phorbol-12-myristate-13-acetate (PMA) and 20 µl of formyl-Met-  
25 Leu-Phe (fMLP) were then added and RLU were recorded at the end of 5 minutes, 20 minutes and 35 minutes after stimulation. Coping capacity was determined by dividing the RLU by the white cell count and the number of minutes of the test and adjusting for background activity and presented as phagocytic capacity per cell per min.

(ii) *Nitro Blue Tetrazolium Test (NBT)*

This test used to determine the 'current' oxidative state of the neutrophils within the sample, as determined by the oxidative capacity of the neutrophils to reduce nitro-blue tetrazolium and form a formazan pigment.

10 10 µl of blood was mixed with 10 µl of NBT, diluted at 1mg per ml, and incubated at 37°C for ten minutes sample was then smeared onto two clean microscope slides and air dried. Samples were then flooded with Accustain Wright stain, rinsed with distilled water and dried. Percentage of 'activated' neutrophils was determined by 10 careful counting under the light microscope.

(iii) *Whole White Cell Count (WCC)*

A manual WCC count was undertaken for each patient. 10 µl of blood was mixed 15 with 90 µl of 2% acetic acid coloured pale purple with crystal violet and gently mixed to lyse the red blood cells. A sample was then placed on a haemocytometer and a careful count was made. A mean WCC per ml was calculated from at least five fillings of the haemocytometer.

20 (iv) *Measures*

Each patient completed the ten-item Perceived Stress Scale (Cohen, Kamarck & Mermelstein, 1983), the 14-item Hospital Anxiety Depression Scale (Zigmond & Snaith) and the 20-adjective, Positive and Negative Effect Scale (PANS) (Watson, Clark & Tellegen 1988). In addition, a six-item visual analogue scale was constructed 25 to investigate the perception of stress specifically about the illness, the self-efficacy of the patient and their locus of control. The items from this scale are listed below:

1. How 'stressed' do you feel about you health Right Now?
2. How much control do you feel you have over you health in general?

3. How much control do you feel you have over your heart condition Right Now?
4. Overall how confident are you that your heart condition will have improved in 12 months time?
5. How much control do you feel health professionals have over your heart condition Right Now?
6. How confident are you that you can do all the things necessary to manage your condition and/or its treatments on a regular basis?

Each item could be scored on a scale from 0 (no stress, no control, not confident) to 10 (stress as bad as it can be, complete control, completely confident).

Clinical pre-operative data was also recorded for all patients including previous infarct; angina status, lung function and grading by the anaesthetist (ASA grade) (data obtained from hospital record database).

15

### Results

#### **(i) Summary**

Results revealed a consistent, statistically significant association between stress and %NBT cells pre and post-surgery ( $p < 0.001$ ) in both groups. There was a significant difference in perceived stress ( $p = 0.009$ ) between the groups, pre-surgery, both significantly reduced post-surgery. No significant differences were found between or within the groups for anxiety, depression, and positive and negative effect. Interestingly, the 'acute' group had the highest perceived psychological stress as determined by self-administered questionnaires. However, coping capacity as determined from blood samples was significantly greater in the 'acute' group both before and after surgery ( $p < 0.02$ ).

*(ii) Perceived stress, anxiety and the current activity (NBT) of neutrophils.*

**(a) Pre-Surgery**

A consistent and significant relationship between PSS and percentage of NBT positive neutrophils was found in both groups ( $r = 0.912, p = 0.0001$  in the elective group and  $r = 0.878, p = 0.0001$  in the acute group).

There were also found consistent and significant relationships in both groups between the percentages of NBT positive neutrophils and anxiety ( $r = 0.670, & r = 0.616, p = 0.0001$ ), depression ( $r = 0.632, & r = 0.664, p = 0.0001$ ), negative effect ( $r = 0.601, p = 0.0001 & r = 0.581, p = 0.001$ ) and dyspnoea status ( $r = 0.275, p = 0.028 & r = 0.399, p = 0.044$ ). There was also found a significant relationship between NBT and health related stress within the elective group ( $r = 0.499, p = 0.0001$ ) but not in the acute group.

**15 (b) Post Surgery**

A consistent and significant relationship between PSS and percentage of NBT positive neutrophils was found in both groups ( $r = 0.851, p = 0.0001$  in the elective group and  $r = 0.944, p = 0.0001$  in the acute group).

20 Consistent and significant relationships were found between the percentage of NBT positive neutrophils and PSS ( $r = 0.851 & r = 0.944, p = 0.0001$ ), anxiety ( $r = 0.488; & 0.864, p = 0.0001$ ), depression ( $r = 0.475, p = 0.0001 & r = 0.688, p = 0.001$ ), positive effect ( $r = -0.329, p = 0.09 & r = -0.593, p = 0.006$ ), negative effect ( $r = 0.494, & r = 0.831, p = 0.0001$ ) and health related stress ( $r = 0.456, & r = 0.707, p = 0.0001$ ).

The above results, from both the pre and post surgery time points, suggest that the percentage of NBT positive neutrophils show considerable associations with the psychological state of the patients studied with the negative states (e.g. anxiety,

depression, PSS, negative effect and health related stress) increasing as the percentage of NBT neutrophils increases. Positive effect shows a negative association suggesting that as positive effect increases the percentage of NBT positive neutrophils decreases. However few or no associations are seen between 5 NBT and disease indicators.

### (iii) *Coping capacity of neutrophils*

Analysis of the chemiluminescence data (Mann-Whitney U test) comparing the groups at each time-point revealed no significant differences between the groups at 5 minutes or 20 minutes for pre- or post-surgery, samples (see Tables 5 and 6 below). 10 However, a consistent and significant difference in coping capacity was found at 35 minutes for both pre- ( $p = 0.018$ ) and post-surgery ( $p = 0.019$ ) (see Figures 6 and 7).

Table 5: Comparison of the phagocytic capacity at the three time points (pre-surgery)

Elective Group				Acute Group			
	$n = 64$				$n = 26$		
Time	median	max	min	median	max	min	p
5 mins.	0.00743	0.2112	-0.0011	0.00775	0.0194	-0.0027	ns
20 mins.	0.01547	0.0967	0.0017	0.17423	0.0684	0.0026	ns
35 mins.	0.35613	0.5069	0.0065	0.05610	0.1679	0.0153	0.018

15

Table 6: Comparison of the phagocytic capacity at the three time points (post-surgery)

Elective Group				Acute Group			
	$n = 62$				$n = 20$		
Time	median	max	min	median	max	min	p
5 mins.	0.00191	0.0226	-0.0042	0.00599	0.0123	-0.0011	ns
20 mins.	0.01701	0.0883	-0.0123	0.01954	0.1086	0.0028	ns
35 mins.	0.03299	0.1677	-0.0163	0.04368	0.1137	0.0224	0.019

Bivariate correlations of the data show no significant associations between coping capacity and either the psychological variables or the physiological indicators.

5     *(iv) Perceived stress and health related stress.*

The level of PSS reported in both groups prior to surgery was significantly higher than post surgery ( $t = 2.72$ ,  $p = 0.008$  &  $t = 3.44$ ,  $p = 0.001$ ). It is also clear from the mean scores that the acute group were reporting significantly higher levels of PSS than the elective group ( $t = -2.674$ ,  $p = 0.009$ ). This is also true of health related stress which was significantly higher pre-surgery than post-surgery in both groups ( $t = 5.19$ ,  $p = 0.000$ , &  $t = 3.76$ ,  $p = 0.001$ ). Again, the highest mean score is in the acute group pre surgery. There were no significant differences between the health related stress scores between the groups pre-surgery. The 'norm' PSS for this population was  $12 \pm 6.3$  (SD) compared to slightly higher in the elective group (14.72,  $\pm 7.21$ ), but much higher in the acute group (19.12,  $\pm 6.71$ ), suggesting that the acute group was the more 'stressed'. Post surgery scores were near to population 'norms'.

10     *(v) Conclusion*

15     Whilst patients from the acute group perceived themselves to be more stressed than patients from the elective group, their leucocytes had a greater coping capacity than those from the elective group. In physiological terms they would be less susceptible to opportunistic infections and stress-related illness.

CLAIMS

1. A method for determining whether an individual, which is a mammal or bird, is experiencing changed physiological status arising from exposure to a psychological stressor, the method comprising:
  - 5 (a) contacting a test sample comprising neutrophils obtained from said individual with an inducer capable of stimulating superoxide production in neutrophils, under conditions suitable for such stimulation;
  - (b) determining the increase in superoxide production above basal in said test sample after a time period when neutrophils of the same species in a control sample, which are free or substantially free of stress-induced activation or at least derived from one or more individuals exposed to the same regime minus a factor to be tested as a psychological stressor, will exhibit superoxide production under the same *in vitro* conditions; and
  - 10 (c) comparing the increase in superoxide production above basal observed in said sample with the increase in superoxide production above basal observed in a control sample as defined in (b) above under the same conditions;

15 wherein lower superoxide production in said test sample is indicative of the effect of a psychological stressor on the individual's physiological status.
2. A method according to claim 1 for determining the coping capacity of an individual for exposure to a psychological stressor, wherein prior to step (a) said individual is exposed to said psychological stressor for a time period whereby neutrophils in an individual of the same species who is susceptible to stress induced by said stressor will exhibit increased superoxide production and wherein the degree of further *in vitro* induced superoxide production in said test sample above basal determined in step (c) is a measure of coping capacity.
- 30 3. A method according to claim 1 or claim 2 wherein said sample comprises isolated leucocytes

4. A method according to claim 1 or claim 2 wherein said sample is a whole blood sample.
  5. 5. A method according to any one of claims 1 to 4, wherein the individual is human.
  6. A method according to any one of claims 1 to 4 wherein the individual is a bird, such as a chicken.
- 10
7. A method according to any one of claims 1 to 4 wherein the individual is a farmed animal, such as a cow, pig, sheep, lamb or poultry.
  8. A method according to any one of claims 1 to 4 wherein the individual is a wild mammal.
- 15
9. A method according to any of the preceding claims, wherein the inducer capable of stimulating superoxide production in neutrophils is phorbol myristate acetate (PMA), N-Formyl-Met-Leu-Phe (fLMP chemotactic peptide), zymosan, lipopolysaccharide or adrenaline.
- 20
10. A method according to any of the preceding claims, wherein superoxide production is detected using luminol or isoluminol as an amplifier and the resulting chemiluminescence is measured.
- 25
11. A method according to claim 1 or claim 2, wherein the inducer capable of stimulating superoxide production in neutrophils is phorbol myristate acetate (PMA), superoxide production is detected using luminol as an amplifier and the resulting chemiluminescence is measured.
- 30
12. A method of screening for a stress-relieving drug, the method comprising:
    - (a) administering a test compound to an individual;

- (b) exposing said individual to a psychological stressor and measuring their coping capacity using a method according to claim 2; and
- (c) comparing their coping capacity after administration of the test compound to their coping capacity in the absence of the test compound, wherein an increase in coping capacity after administration of the test compound is indicative of stress-relieving ability of said test compound.
- 5
13. A method according to claim 12, wherein the individual is a non-human mammal.
- 10
14. A method according to claim 12 or claim 13, further comprising synthesizing a stress-relieving drug identified by said method, and/or formulating the drug into a pharmaceutical composition.
- 15
15. Use of a stress-relieving drug in the manufacture of a medicament for treating an individual who has been identified as suffering stress using a method according to any of claims 1 to 11.
- 20 16. A method of treating an individual suffering from stress which comprises providing a stress-relieving treatment, such as administering a stress-relieving drug, to an individual identified as suffering from stress using a method according to any one of claims 1 to 11.
- 25 17. A method of testing the efficacy of a proposed stress-relieving treatment which comprises exposing an individual to a psychological stressor in the presence and absence of said treatment and determining their coping capacity in accordance with any one of claims 2 to 11.
- 30 18. A method as claimed in claim 1 wherein the control sample is replaced by a test sample comprising neutrophils taken from a second individual, the test samples being taken from different individuals at the same time point before,

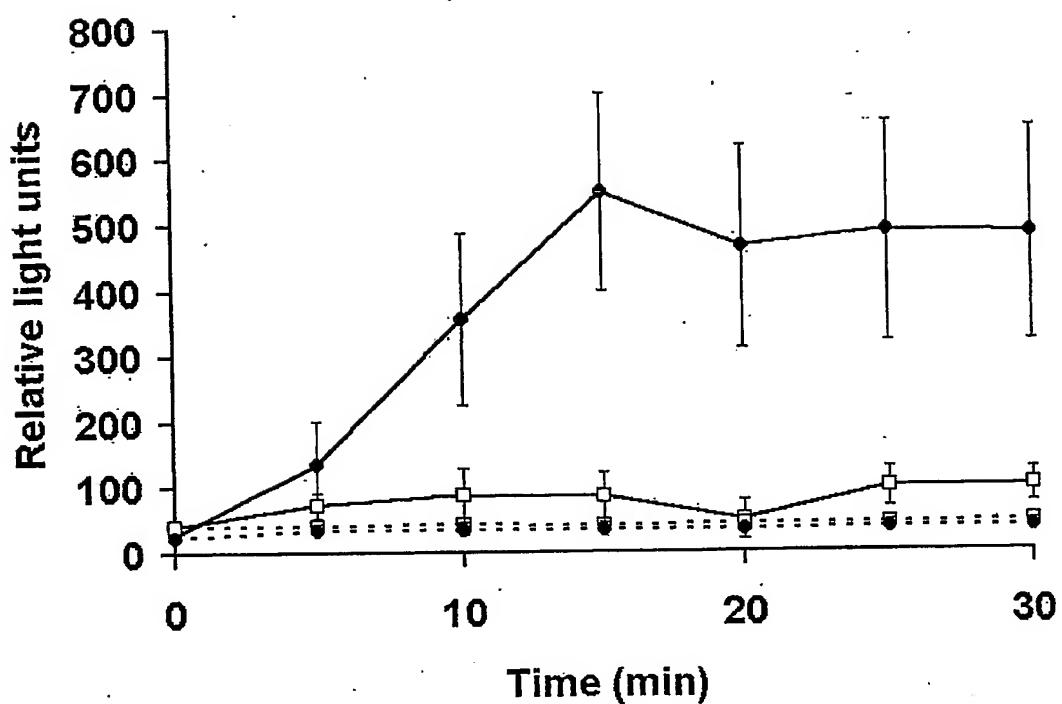
during, or after subjection to two different regimes to be compared as stressors, and wherein the superoxide production above basal determined for each sample is adjusted to take account of differences in white cell count or neutrophil count in the samples.

5

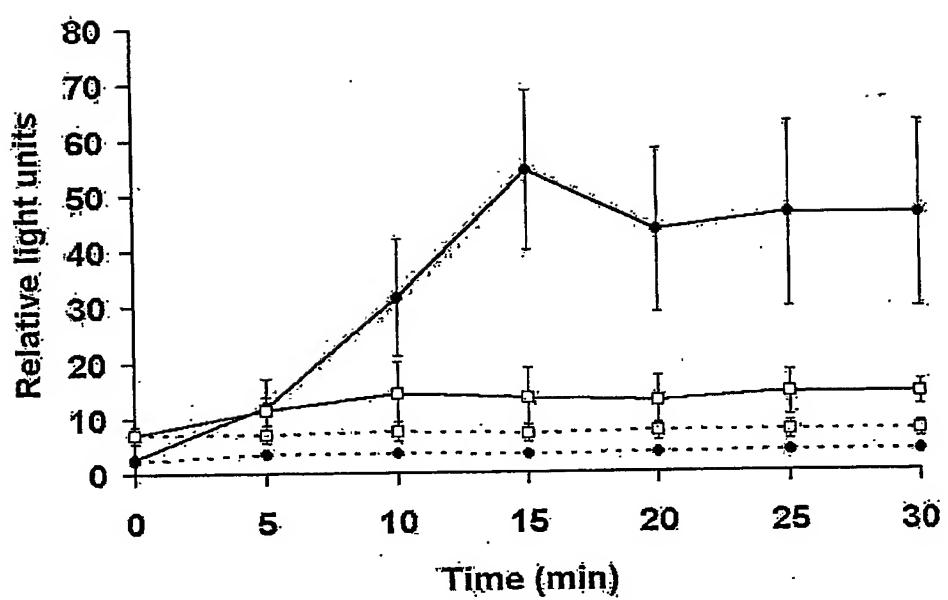
19. A method for comparing two regimes as stressors wherein a method according to claim 18 is repeated at several time points employing samples from more than one individual subjected to each regime.
- 10 20. A method as claimed in claim 18 or claim 19 wherein the regimes to be compared are protocols for medical treatment.
- 15 21. A method as claimed in claim 18 or claim 19 wherein the regimes to be compared are a known stressful regime plus and minus a proposed stress-alleviating treatment.
22. A device for carrying out a method according to claim 1 comprising a portable chemiluminometer and system for analysing the results to provide a stress measurement.

20

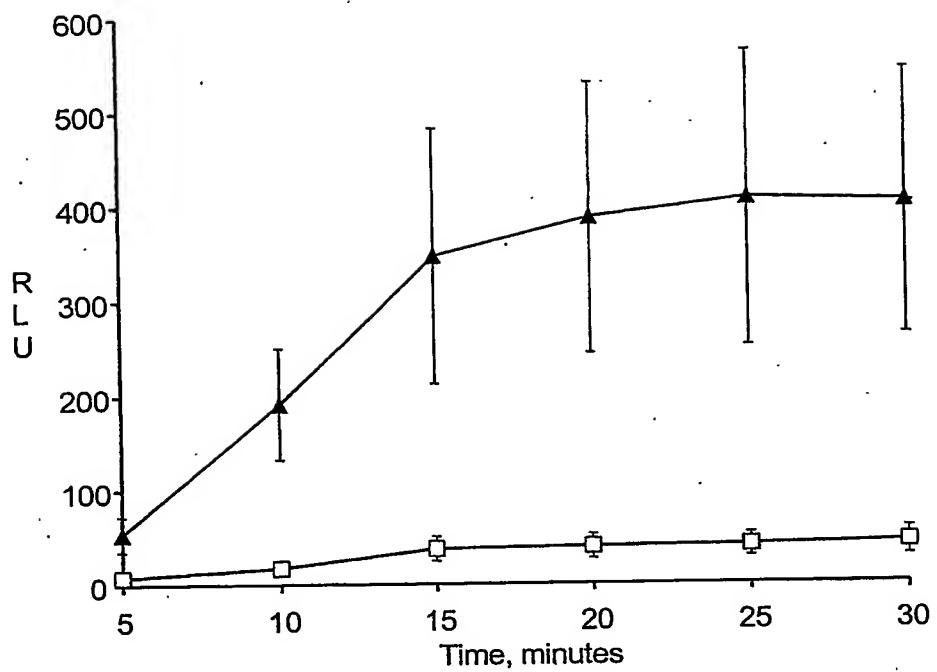
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**Figure 1**

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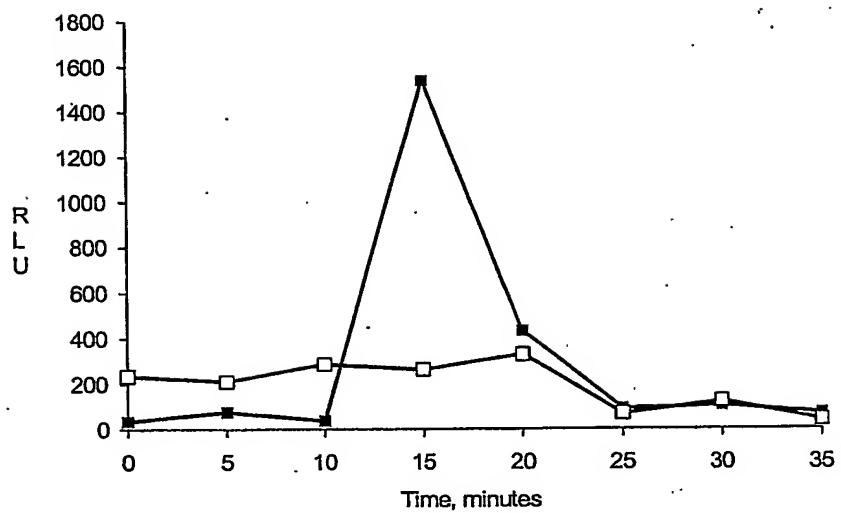
**Figure 2**

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**Figure 3**

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Figure 4



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Figure 5(a)

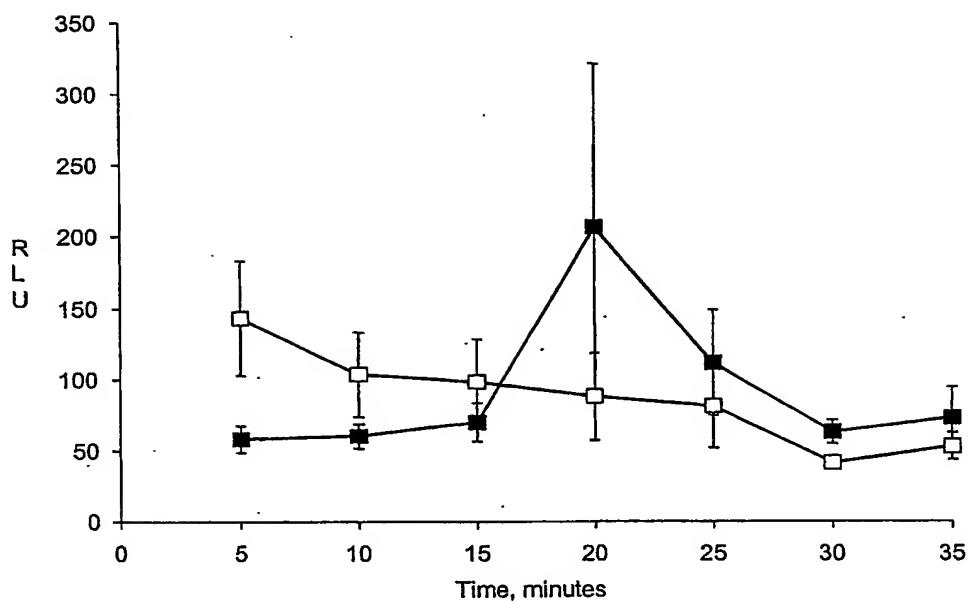
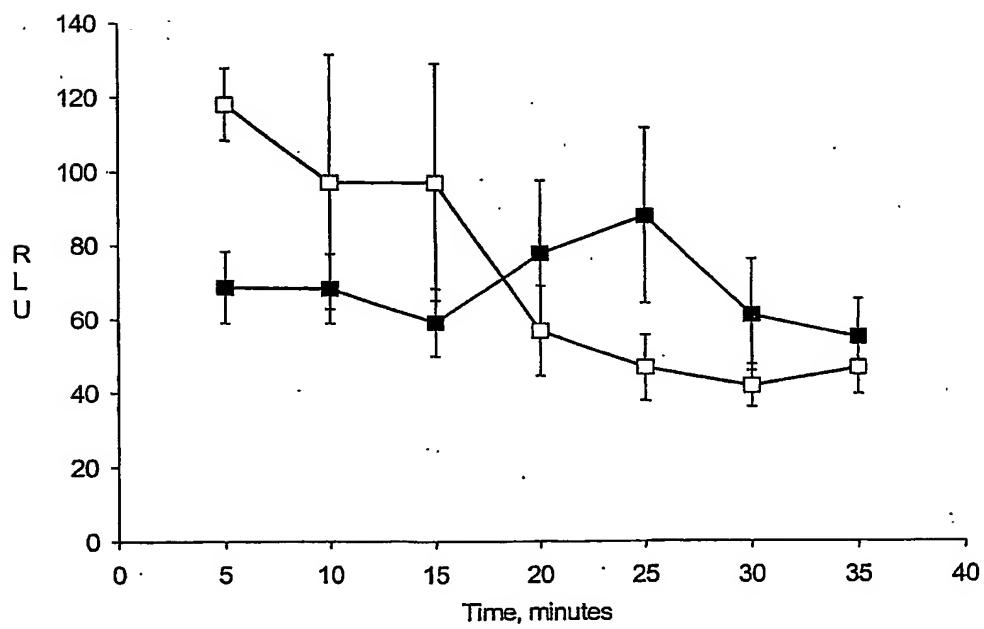


Figure 5(b)



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Figure 6

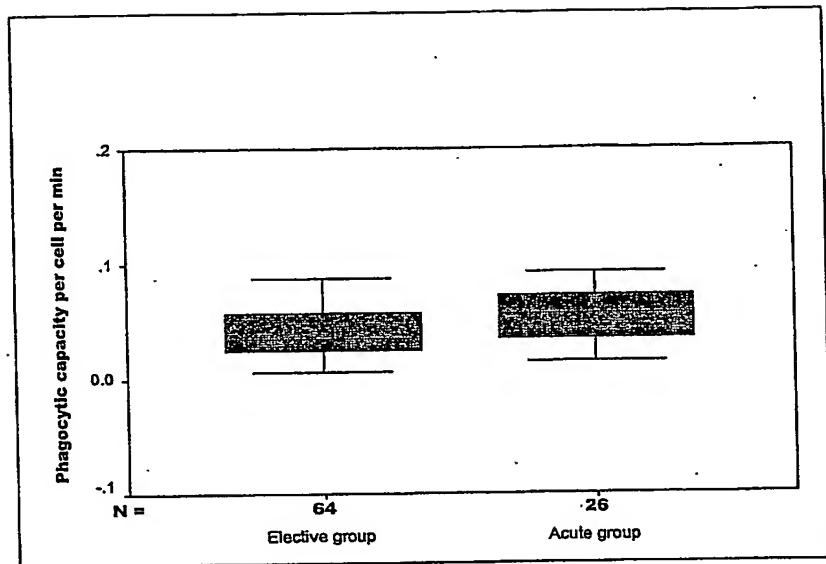
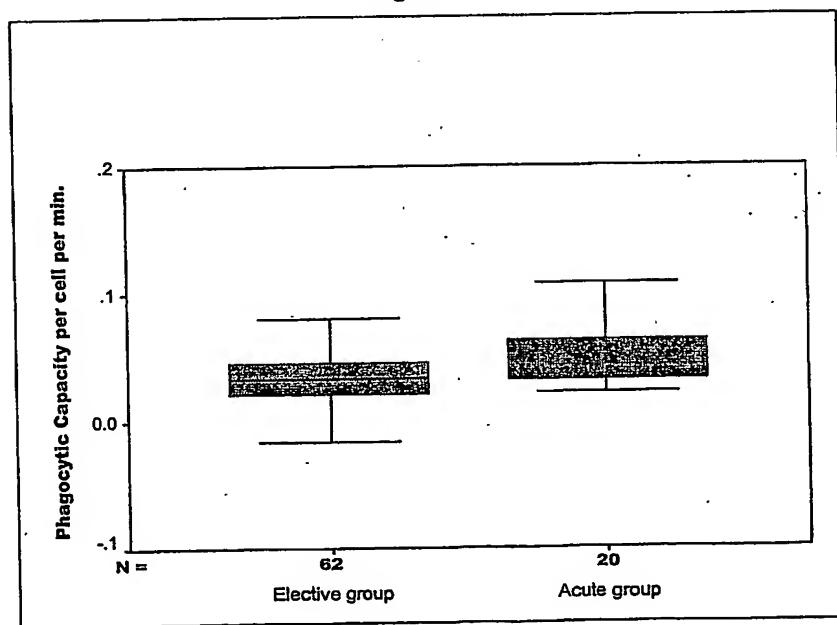


Figure 7



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 03/04749

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	EP 1 262 167 A (COGNIS FRANCE S A) 4 December 2002 (2002-12-04) page 2, paragraph 2 page 15, paragraph 4 —	22
X	US 5 492 816 A (PFEFFERKORN LORRAINE C) 20 February 1996 (1996-02-20) abstract example 6 —	22 —/—

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the International search

Date of mailing of the International search report

24 February 2004

12/03/2004

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Weijland, A

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 03/04749

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DEFAUW JEAN M ET AL: "Synthesis and protein kinase C inhibitory activities of acyclic balanol analogs that are highly selective for protein kinase C over protein kinase A"</p> <p>JOURNAL OF MEDICINAL CHEMISTRY, vol. 39, no. 26, 1996, pages 5215-5227, XP002177939 ISSN: 0022-2623 abstract page 5227, left-hand column, paragraph 4</p> <p>---</p>	1-14, 17-22
A	<p>AZUMA YASUTAKA ET AL: "Endorphins delay constitutive apoptosis and alter the innate host defense functions of neutrophils"</p> <p>IMMUNOLOGY LETTERS, vol. 81, no. 1, 1 April 2002 (2002-04-01), pages 31-40, XP002271166 ISSN: 0165-2478 the whole document</p> <p>---</p>	1-14, 17-22
Y	<p>KANG D H ET AL: "The effect of psychological stress on neutrophil superoxide release."</p> <p>RESEARCH IN NURSING &amp; HEALTH. UNITED STATES OCT 1994, vol. 17, no. 5, October 1994 (1994-10), pages 363-370, XP009026477 ISSN: 0160-6891 abstract</p> <p>---</p>	1-14, 17-22
A	<p>FERRÁNDEZ M D ET AL: "Effects of a long-term training program of increasing intensity on the immune function of indoor Olympic cyclists."</p> <p>INTERNATIONAL JOURNAL OF SPORTS MEDICINE. GERMANY NOV 1996, vol. 17, no. 8, November 1996 (1996-11), pages 592-596, XP009026478 ISSN: 0172-4622 the whole document</p> <p>---</p>	1-14, 17-22

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 03/04749

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: 15-16 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 15-16

Present claims 15-16 relate to an extremely large number of possible methods, since they include in the methods stress-releasing drugs obtained from a method of screening. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to claims 1-14 and 17-22.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB 03/04749

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 1262167	A 04-12-2002	EP WO	1262167 A1 02098385 A1	04-12-2002 12-12-2002
US 5492816	A 20-02-1996	US	5401640 A	28-03-1995